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RESEARCH ARTICLE

Genetic relationships of hellbenders in the Ozark highlands of Missouri and conservation implications for the Ozark subspecies (*Cryptobranchus alleganiensis bishopi*)

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Abstract The hellbender (*Cryptobranchus alleganiensis*) is an obligately aquatic salamander that is in decline due to habitat loss and disease. Two subspecies of hellbender have been described based on morphological characteristics: *C. a. alleganiensis* (eastern subspecies) and *C. a. bishopi* (Ozark hellbender). Current conservation strategies include captive propagation for restorative releases even though information regarding the current levels of genetic variability and structure within populations is not sufficient to effectively plan for conservation of the genetic diversity of the species. To investigate patterns of population structure in the hellbender, we genotyped 276 hellbenders from eight Missouri River drainages, representing both subspecies. Our results showed low levels of within-drainage diversity but strong population structure among rivers, and three distinct genetic clusters. F_{ST} values ranged from 0.00 to 0.61 and averaged 0.40. Our results confirmed previous reports that *C. a. bishopi* and *C. a. alleganiensis* are genetically distinct, but also revealed an equidistant relationship between two groups within *C. a. bishopi* and all populations of *C. a. alleganiensis*. Current subspecies delineations do not accurately incorporate genetic structure, and for conservation purposes, these three groups should be considered evolutionarily significant units.

Keywords Amphibian · Salamander · Population genetics · Microsatellites · *Cryptobranchus*

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Introduction

The goal of many conservation genetics studies is to gain a better understanding of the ecological and evolutionary forces that produce patterns of population structure across time and space. Current population structure reflects both historical events, such as range fragmentation or expansion, and current levels of connectivity and gene flow. For species of conservation concern, studies of population structure can help identify populations that are currently isolated and may be subject to the deleterious effects of inbreeding. Managers can use this information to inform conservation planning and to define management units (Moritz 1994; Crandall et al. 2000).

Cryptobranchids are unique relative to other salamanders because of their primitive body design and large size (Nickerson and Mays 1973a; Petranka 1998). There are three extant species: *Andrias davidianus* (Chinese giant salamander), *A. japonicus* (Japanese giant salamander), and *Cryptobranchus alleganiensis* (North American hellbender). All are obligately aquatic and are experiencing population declines due to habitat adulteration and loss, environmental contamination, and illegal harvest for consumption or the exotic pet trade (Trauth et al. 1992; Wheeler et al. 2003; Wang et al. 2004).

Cryptobranchus alleganiensis is composed of two subspecies that have been described based on morphological differences and, to some extent, geographic location. *C. a. alleganiensis*, the eastern hellbender, has the largest range, historically occupying the eastern United States from New York to Missouri and south to Georgia. It is the larger of the two subspecies and exhibits small dorsal spots and more uniform chin coloration (Nickerson and Mays 1973a). *C. a. bishopi*, the Ozark hellbender, inhabits the Ozark highlands of southern Missouri and northeastern Arkansas (Nickerson and Mays 1973a; Petranka 1998). It has smaller spiracles, a smoother lateral line system in the pectoral area, dark patchiness under the chin, and dorsal blotching (Nickerson and Mays 1973a). The Ozark hellbender attains an average length of 29–57 cm (Dundee and Dundee 1965), while the eastern hellbender may measure 45–60 cm (Wheeler et al. 2003). Missouri is the only state in which both taxa occur, although within the state they do not overlap in range. *C. a. bishopi* occurs only in the Black and North Fork of the White River system, which flow southward, while *C. a. alleganiensis* is found in streams draining to the north as part of the Missouri and Meramec River basins. Surveys of five Missouri Rivers have shown that juvenile recruitment is decreasing and populations of both hellbender subspecies have declined by 70–80% in the last 20 years (Wheeler et al. 2003). Population sizes in Missouri are estimated to be low: 380 Ozark hellbenders and 600 of the eastern subspecies (Briggler et al. 2007b). Both subspecies are listed as state endangered in Missouri and the Ozark hellbender is a candidate for federal listing under the U.S. Endangered Species Act.

Hellbenders are considered habitat specialists because they require clear, swiftly flowing streams with a substrate of large rocks (Smith 1907; Bishop 1941; Nickerson and Mays 1973a). Siltation (Trauth et al. 1992; Wheeler et al. 2003), habitat alterations (Wheeler et al. 2003), collection for scientific study (Nickerson and Briggler 2007), and the possible spread of pathogens such as amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Briggler et al. 2007a, 2008) have all been implicated in the decline of this species.

The species' vulnerability to habitat changes is exacerbated by its life history characteristics: hellbenders have a long lifespan of 20–30 years (Taber et al. 1975; Peterson 1979), a slow growth rate, and become sexually mature at the relatively late age of 5–8 years (Smith 1907; Dundee and Dundee 1965; Nickerson and Mays 1973a; Peterson et al. 1988; Petranka 1998). Hellbenders have been shown to be highly sedentary: a three-month mark-recapture study of *C. a. bishopi* showed that 95% of individuals remained within 90 m of the site of capture (Nickerson and Mays 1973b). For these reasons, hellbenders are highly susceptible to localized extinction. Recolonization from adjacent populations is hindered by intervening substrates that are unsuitable for habitation and by aquatic conditions that make dispersal difficult. This leads to isolation and restricted gene flow among populations (Routman 1993).

While conservation programs often involve efforts to reconnect isolated populations or restock using captive bred individuals, hellbender conservation has been hindered by a lack of information regarding the genetic history of the species. Merkle et al. (1977) found low levels of allozyme variation between *C. a. alleganiensis* and *C. a. bishopi* and suggested that subspecific designations were not warranted. Kucuktas et al. (2001) used random amplified polymorphic DNA (RAPD) techniques and found only limited variation within the Ozark subspecies. Mitochondrial DNA differentiation is considerable between drainages but is relatively limited within-drainages (Routman 1993; Routman et al. 1994). Phylogenetic analyses based on mitochondrial DNA revealed a paraphyletic relationship between the subspecies (Sabatino and Routman 2009). Our study objectives were to use genotypic information at multiple microsatellite loci to (1) quantify genetic diversity of Missouri populations of *C. a. alleganiensis* and *C. a. bishopi*, (2) reveal the extent of structure within basins and populations, and (3) delineate management units for conservation efforts that may involve propagation and release of the species.

Methods

Samples and DNA analyses

From 2005 to 2009, a total of 276 hellbender samples were collected from eight drainages in Missouri (Fig. 1; Table 1). Hellbenders were captured during snorkeling surveys, and a small tissue sample was taken from the tip of the tail and preserved in 95% ethanol. Tail-tip clipping has been shown not to adversely affect amphibian body condition or survival (Arntzen et al. 1999). Animals were individually marked with PIT tags and released at their site of capture.

Total genomic DNA was extracted from tissue samples using the DNeasy Blood and Tissue kit™ (Qiagen), following the manufacturer's recommendations. DNA was stored at −60°C. We used the polymerase chain reaction (PCR) to amplify seven microsatellite loci using primers developed for hellbenders by Johnson et al. (2009). Six of the loci (CRAL2, CRAL4, CRAL9, CRAL10, CRAL13, CRAL15) contain dinucleotide repeats, while CRAL17 contains a tetranucleotide repeat region. Amplifications were performed in 15 µl volumes using approximately 15 ng genomic DNA, 1.5 µl Ampli*Taq* Gold 10× reaction buffer (Applied Biosystems, Inc.), 0.2 mM of each dNTP,

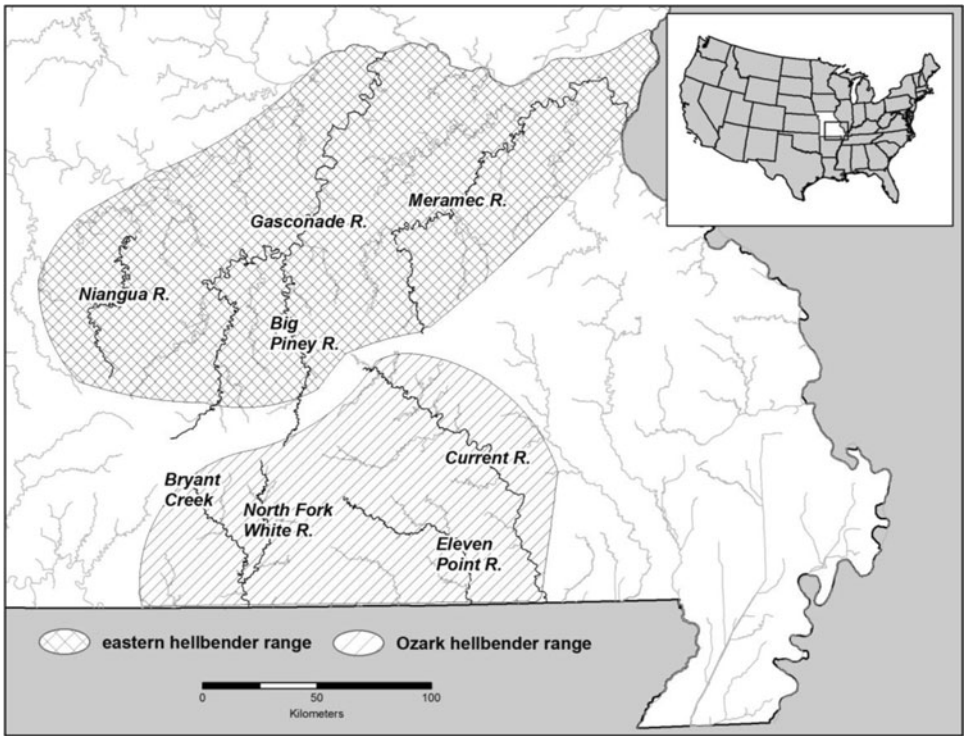

Fig. 1 Location of rivers from which hellbender (*C. alleganiensis*) samples were collected, with ranges of the two subspecies shown

Table 1 Sampling locations and sample sizes for eastern (*C. a. alleganiensis*) and Ozark (*C. a. bishopi*) hellbenders

Subspecies	Drainage	Code	Number of samples
Eastern hellbender, *C. a. alleganiensis*	Big Piney River	BP	40
	Gasconade River	GA	45
	Meramec River	ME	8
	Niangua River	NI	45
Ozark hellbender, *C. a. bishopi*	Bryant Creek	BC	9
	Current River	CU	39
	Eleven Point River	EP	45
	North Fork of White River	NF	45

0.5 μM of each primer, 2 mM $MgCl_2$, 1 μl bovine serum albumen (BSA), and 0.5 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems, Inc.). The forward primer of each pair was 5′-labeled with a fluorescent dye (VIC, NED, Applied Biosystems, Inc.; or 6-FAM, Sigma). One sample was used as a positive control for all reactions to standardize allele size scoring among amplifications. A negative control was also included with each reaction to detect possible contamination of reagents. Cycling conditions were as reported in Johnson et al. (2009). Reactions were performed in an Eppendorf Mastercycler® ep thermocycler; to verify amplification, 5 μl PCR product was electrophoresed in a 2% agarose gel prestained with GelStar (Lonza). Amplification products were analyzed in an ABI 3730 DNA Analyzer (Applied Biosystems, Inc.) at the University of Missouri DNA Core Facility. Allele sizes were determined using Genemarker 1.6 (Softgenetics, LLC) by comparison with the LIZ 600 sizing standard. Samples that were difficult to score or appeared aberrant were reanalyzed using new amplification products.

Statistical analyses

We used Genepop 3.4 (Raymond and Rousset 1995) to test for linkage disequilibrium using a Markov Chain approximation and to test for deviations from heterozygosity values expected under Hardy–Weinberg Equilibrium (HWE). Allele frequencies and expected and observed heterozygosities were calculated for each locus and population using Genepop. To compensate for multiple comparisons, a Bonferroni correction was used to maintain an overall significance value of 0.05.

Arlequin 3.11 (Excoffier et al. 2005) was used to calculate estimated pairwise F_{ST} (Weir and Cockerham 1984) and R_{ST} (Rousset 1996) values for all population pairs at each locus, and permutation tests were used to calculate

significance levels for the values obtained. Because allelic diversity measures are sensitive to sample size, rarefaction was performed in HP-RARE 1.0 (Kalinowski 2005) to determine total and private allelic diversity at a sample size of eight (the minimum number of samples collected from any drainage). Using these diversity values we employed an ANOVA to test for differences in allelic richness among populations and a *t* test to detect differences in allelic richness between the eastern and Ozark subspecies. We used MICRO-CHECKER 2.2.1 (van Oosterhout et al. 2004) to test for evidence of large allele dropout and genotyping error due to stutter, to test for the presence of null alleles at each locus, and to estimate their frequency within each drainage. In MICRO-CHECKER, the confidence interval for detecting errors and null alleles was 95% with a Bonferroni correction.

To detect population structure based on individual genotypes, we used STRUCTURE version 2.3.2 (Pritchard et al. 2000). Ten replicate runs consisted of 10,000 iterations for simulation burnin followed by 100,000 iterations for each K from 1 to 10. All samples were analyzed such that STRUCTURE had no prior information regarding sample locations. From the ten runs, we calculated the mean and standard deviation of the estimated likelihood for each value of K. To determine the most likely number of population clusters, we also used the delta K statistic (Evanno et al. 2005). After using STRUCTURE to identify the first level of hierarchy (three distinct clusters, see results below) we ran each genetic cluster separately, to examine second order structure. We used AMOVA tests in ARLEQUIN to examine the distribution of genetic variation at three hierarchical levels: within groups, defined either as subspecies or as the three genetic clusters identified in STRUCTURE; among populations within groups; and within populations. Significance of the AMOVA results was assessed using permutation tests.

We used GENECLASS2 (Piry et al. 2004) to calculate individual assignment probabilities based on a Bayesian framework (Rannala and Mountain 1997) and an assignment threshold of $P < 0.05$. This program generates 10,000 random genotypes from the data, and assigns an individual sample to the reference population in which the likelihood of its genotype is maximized.

Results

We used seven microsatellite loci to genotype 138 individuals of *C. a. alleganiensis* and 138 individuals of *C. a. bishopi* (Table 1). There was no evidence of linkage disequilibrium between any pair of loci in any population. Average expected heterozygosity (H_e) levels ranged from 0.31 to 0.48 across all populations. Six of 56 (10.7%) tests for deviations from Hardy–Weinberg equilibrium (HWE) were significant after a Bonferroni correction (Table 2). At locus CRAL2, three of the four populations of eastern hellbenders deviated significantly from HWE, with fewer heterozygotes than expected.

The total number of alleles across all populations ranged from 2 to 20 per locus with an average of 11.14 (±7.18 SD). The average number of alleles per locus was highest in the Current, Eleven Point, and Gasconade Rivers, and lowest in Bryant Creek and the Meramec River. Private alleles per population ranged from zero for the North Fork of the White River, Bryant Creek, and Big Piney River to twelve within the Current River population (Table 2).

When compared using rarefaction at a sample size of eight for each population, there were no significant differences in allelic richness (ANOVA, df = 7, $P = 0.94$) or private allelic richness (ANOVA, df = 7, $P = 0.20$) among populations. When populations were grouped by subspecies, there were also no significant differences in allelic richness (Ozark = 2.50 ± 1.84; eastern = 2.71 ± 1.84; two tailed *t* test, df = 6, $P = 0.77$) or private allelic richness (Ozark = 0.41 ± 0.11; eastern = 0.47 ± 0.24; two-tailed *t* test, df = 6, $P = 0.81$) (Table 2).

MICRO-CHECKER suggested the presence of null alleles at CRAL2 in three of the four eastern hellbender populations, with frequencies ranging from 0.13 to 0.24 (Table 2). In Ozark hellbender populations, the program suggested the presence of null alleles at CRAL4 in three populations (frequency range 0.12–0.19) and at CRAL9 in two populations (frequency of 0.19 in both). Since CRAL2 appeared to have null alleles in three of the four eastern hellbender populations and differed significantly from expectations in the fourth, that locus was eliminated from the analysis of genetic distances among populations.

F_{ST} and R_{ST} values indicate substantial differentiation among Ozark populations and between Ozark and eastern hellbender populations (Table 3). In the eastern hellbender populations, F_{ST} values indicate that only the Big Piney River and the Gasconade do not differ significantly. Within the Ozark subspecies, F_{ST} values indicate significant differentiation between all population pairs except the North Fork of the White River and Bryant Creek. With the exception of Bryant Creek, F_{ST} values between North Fork of the White River and other Ozark populations were as great as F_{ST} values between Ozark and eastern populations. All eastern populations differed significantly from Ozark populations (Table 3). The R_{ST} measure, which is based on the stepwise mutation model and may thus be more applicable to microsatellite loci than F_{ST}, suggested patterns that were similar to those provided by F_{ST}, although population differentiation values were greater under R_{ST}.

When evaluated using the delta K statistic (Table 4), STRUCTURE identified three distinct population groupings

A null allele represents a dysfunctional protein or an absent allele

Table 2 Genetic variation in eastern (*C. a. alleganiensis*) and Ozark hellbenders (*C. a. bishopi*)

Gasconade ($n = 45$)						Big Piney ($n = 40$)					Niangua ($n = 45$)					Meramec ($n = 8$)				
Locus	A(Ap)	Ar(Arp)	He	Ho	Null	A(Ap)	Ar(Arp)	He	Ho	Null	A(Ap)	Ar(Arp)	He	Ho	Null	A(Ap)	Ar(Arp)	He	Ho	Null
Eastern hellbender (*C. a. alleganiensis*)																				
Cral2	13(0)	5.7(0.6)	***0.89***	***0.65***	0.13	10(0)	5.0(0.3)	***0.85***	***0.72***	–	16(3)	5.6(1.3)	***0.87***	***0.55***	0.18	5(0)	4.1(0.1)	0.79	0.38	0.24
Cral4	6(1)	2.4(0.1)	0.51	0.47	–	2(0)	1.9(0.0)	0.40	0.35	–	3(0)	2.3(0.0)	0.54	0.52	–	1(0)	1.0(0.0)	0.00	0.00	–
Cral9	3(0)	1.8(0.1)	0.26	0.24	–	3(0)	1.9(0.1)	0.29	0.25	–	2(1)	1.8(0.8)	0.28	0.29	–	4(1)	3.0(0.7)	0.46	0.33	–
Cral10	4(2)	2.2(0.3)	0.43	0.44	–	2(0)	1.7(0.0)	0.25	0.23	–	3(0)	2.4(0.0)	0.45	0.47	–	3(0)	2.7(0.1)	0.57	0.25	–
Cral13	4(0)	2.4(0.0)	0.50	0.52	–	4(0)	2.6(0.0)	0.49	0.53	–	2(0)	1.7(0.0)	0.22	0.25	–	2(0)	1.6(0.0)	0.14	0.14	–
Cral15	1(0)	1.0(0.0)	0.00	0.00	–	2(0)	1.3(0.0)	0.07	0.08	–	2(0)	1.5(0.0)	0.15	0.16	–	2(0)	1.8(0.1)	0.26	0.29	–
Cral17	9(0)	4.2(0.3)	0.77	0.64	–	6(0)	3.2(0.2)	0.64	0.60	–	7(0)	4.6(0.0)	0.82	0.73	–	6(0)	4.4(0.3)	0.81	0.75	–
Avg	5.7(0.4)	2.8(0.2)	0.48	0.42		4.1(0)	2.5(0.1)	0.43	0.39		5.0(0.6)	2.8(0.3)	0.48	0.42		3.3(0.1)	2.7(0.2)	0.45	0.31	
North Fork of the White River ($n = 45$)						Eleven Point ($n = 45$)					Bryant Creek ($n = 9$)					Current ($n = 39$)				
Locus	A(Ap)	Ar(Arp)	He	Ho	Null	A(Ap)	Ar(Arp)	He	Ho	Null	A(Ap)	Ar(Arp)	He	Ho	Null	A(Ap)	Ar(Arp)	He	Ho	Null
Ozark hellbender (*C. a. bishopi*)																				
Cral2	8(0)	3.5(0.2)	0.68	0.76	–	12(0)	5.1(0.9)	0.86	0.87	–	3(0)	2.4(0.1)	0.58	0.44	–	9(1)	5.0(0.7)	0.86	0.85	–
Cral4	2(0)	1.8(0.4)	0.31	0.16	0.19	3(1)	1.3(0.2)	0.09	0.04	0.12	2(0)	1.5(0.1)	0.13	0.13	–	2(0)	1.2(0.0)	0.05	0.00	0.15
Cral9	2(0)	1.3(0.2)	***0.09***	***0.00***	0.19	2(0)	1.3(0.2)	***0.09***	***0.00***	0.19	1(0)	1.0(0.0)	0.00	0.00	–	1(0)	1.0(0.0)	0.00	0.00	–
Cral10	4(0)	2.2(0.0)	0.52	0.56	–	8(1)	4.2(1.3)	0.78	0.68	–	3(0)	2.4(0.0)	0.58	0.78	–	8(3)	3.7(1.4)	0.69	0.75	–
Cral13	3(0)	2.1(0.4)	***0.52***	***0.95***	–	5(1)	2.5(0.4)	0.51	0.67	–	2(0)	1.8(0.7)	0.20	0.20	–	4(1)	2.0(0.2)	0.26	0.28	–
Cral15	2(0)	1.8(0.1)	0.28	0.33	–	1(0)	1.0(0.0)	0.00	0.00	–	1(0)	1.0(0.0)	0.00	0.00	–	1(0)	1.0(0.0)	0.00	0.00	–
Cral17	7(0)	3.7(0.1)	0.68	0.67	–	9(0)	4.2(1.0)	0.77	0.80	–	5(0)	3.6(0.0)	0.71	0.78	–	18(7)	6.2(2.9)	0.92	0.90	–
Avg	4.0(0)	2.3(0.2)	0.44	0.49		5.7(0.4)	2.8(0.4)	0.44	0.44		2.4(0)	2.0(0.1)	0.31	0.33		6.1(1.7)	2.9(0.7)	0.40	0.40	

Total (A) and private (Ap) allelic richness, corrected total allelic richness (Ar) and corrected private allelic richness (Arp), estimated using rarefaction at a sample size of $n = 8$, are shown for each population at each locus. Expected (He) and observed (Ho) heterozygosity values and estimated frequencies of null alleles are also shown. Observed heterozygosity values that differ significantly from those expected under Hardy–Weinberg equilibrium are shown in bold italics

Table 3 Genetic distance values for hellbender population pairs computed without locus CRAL2

	NF	BP	NI	CU	GA	EP	ME	BC
NF	–	0.51***	0.49***	0.47***	0.48***	0.48***	0.50***	**0.01**
BP	0.70***	–	0.14***	0.60***	**0.00**	0.61***	0.16***	0.50***
NI	0.73***	0.51***	–	0.53***	0.08***	0.53***	0.13***	0.44***
CU	0.72***	0.71***	0.55***	–	0.56***	0.04***	0.61***	0.51***
GA	0.64***	**0.01**	0.39***	0.69***	–	0.57***	0.13***	0.46***
EP	0.85***	0.85***	0.64***	0.20***	0.81***	–	0.61***	0.52***
ME	0.61***	0.27**	0.14**	0.50***	0.12*	0.73***	–	0.55***
BC	**0.08**	0.67***	0.61***	0.56***	0.57***	0.79***	0.42***	–

River abbreviation codes are as listed in Table 1. Pairwise F_{ST} values are reported above the diagonal, R_{ST} values are below. Significance levels, as determined in ARLEQUIN, are denoted as: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Non–significant values are in bold

(Fig. 2a): all eastern hellbender samples were assigned to one cluster, and the Ozark samples were subdivided into the North Fork of the White River/Bryant Creek cluster and the Eleven Point River/Current River cluster. Cluster assignment values were high, with an average individual score of 0.99. When eastern and Ozark samples were analyzed separately for cluster values from K = 1–8, STRUCTURE identified two genetic clusters within each subspecies (Table 4). Ozark samples were divided into a North Fork of the White River/Bryant Creek cluster and a Current River/Eleven Point River cluster, with very little admixture (Fig. 2b) and strong support in the Evanno test (Table 4). Eastern hellbender samples were grouped into a Big Piney/Gasconade River cluster and a Meramec/Niangua River cluster, but with considerable admixture (Fig. 2c) and much weaker support (Table 4). The results of AMOVA tests confirmed that the genetic clusters identified by STRUCTURE explained the distribution of genetic variation better than subspecies designation. Variation among groups was high, but only a small percentage of the variation could be attributed to differences between populations within clusters while differences between populations within subspecies accounted for a much greater percentage of the variation (Table 5).

GENECLASS2 assignment probability scores ranged from 38.1 to 100%, with a mean of 97.7%. Fifty-one of the 138 (37%) eastern hellbender samples were misassigned, although no samples were assigned to the other subspecies, only to other drainages (Table 6). Twelve of the Ozark samples were misassigned (8.7%); these mostly consisted of assignment of North Fork of White River individuals to the Bryant Creek population ($n = 8$). Two Current River samples were incorrectly assigned to the Eleven Point River. These results provide support for the genetic divisions suggested by the STRUCTURE and AMOVA analyses.

Table 4 Evanno statistics for the analysis of hellbender populations in STRUCTURE 2.3.2

K	Avg ln P(D)	SD ln P(D)	delta K
All populations			
1	−5492.9	0.2	n/a
2	−4323.4	59.2	8.2
3	−3638.2	1.6	383.4
4	−3568.8	23.5	2.4
5	−3540.6	21.0	1.0
6	−3516.3	5.9	5.4
7	−3505.7	35.5	1.4
8	−3504.7	27.8	1.4
9	−3483.7	25.6	2.2
10	−3475.8	33.3	n/a
Ozark hellbender populations			
1	−2476.8	1.0	n/a
2	−1784.0	0.4	1592.0
3	−1714.6	0.9	90.5
4	−1725.9	7.1	7.6
5	−1793.0	23.8	3.8
6	−1910.8	57.3	1.7
7	−1991.8	43.9	2.1
8	−2022.9	41.0	n/a
Eastern hellbender populations			
1	−1751.9	0.3	n/a
2	−1679.0	1.9	26.4
3	−1655.6	2.3	22.0
4	−1680.2	24.4	2.8
5	−1777.9	12.7	5.0
6	−1809.4	44.8	1.8
7	−1878.1	46.1	2.1
8	−1949.6	69.1	n/a

Average values of the log likelihood of the data and the standard deviations for the log likelihoods are shown, along with delta K values for each K (number of genetic clusters)

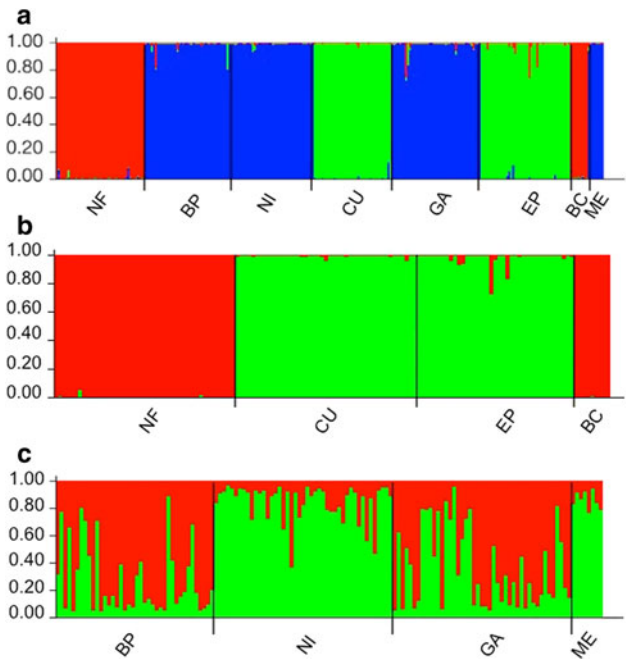

Fig. 2 Results of analysis of genotypes in STRUCTURE 2.3.2. River abbreviation codes are as listed in Table 1. Population structure observed within **a** all sampling locations, **b** rivers in the range of the Ozark hellbender, and **c** rivers in the range of the eastern hellbender

Discussion

Our results demonstrate that hellbender genetic diversity in the Ozark highlands of the central United States is comparable to that of similar salamander species, that there is significant structuring both between basins and within subspecies, and that phylogeographic hypotheses based on subspecies ranges do not accurately reflect genetic subdivisions. These results emphasize that our knowledge of hellbender intraspecific relationships needs to be further refined to facilitate planning and execution of restorative releases. The importance is underscored by the fact that the Spring River (Arkanas) population is now considered extirpated and the North Fork of the White River/Bryant Creek clade is isolated by a large reservoir, eliminating the possibility of recolonization through the White River basin.

Table 6 Results of population assignment tests in GENECLASS2

Source population	Assigned population							
	BP	GA	ME	NI	BC	CU	EP	NF
BP	23	15		2				
GA	23	19		3				
ME		1	6	1				
NI	1	2	3	39				
BC					5			4
CU						37	2	
EP						2	43	
NF					4			41

Genetic variation

Non-transforming salamanders, such as the hellbender, have been characterized as having "extraordinarily low levels" of genetic variation (Shaffer and Breden 1989). An allozyme-based study of genetic diversity in the closely related Chinese giant salamander (*A. davidianus*) reported that 9 of 40 loci were polymorphic but overall heterozygosity was only 0.04 (Murphy et al. 2000). Allozymes in *C. alleganiensis,* based on 137 samples from 12 rivers, were found to be variable at only 2 of 24 loci, leading to the conclusion that limited nuclear variation in the species provided little evidence to maintain subspecific designations (Merkle et al. 1977). The authors hypothesized that paedomorphic salamanders may have less genetic variation than other (metamorphic) salamanders. In contrast, the giant, paedomorphic salamander *Dicamptodon aterrimus* was recently shown to have significant population structure, particularly in relation to catchment, and moderate genetic variability (mean expected heterozygosity: 0.36,

Table 5 Results of hierarchical AMOVA testing of genetic variation within populations, among populations within groups, and among groups

Source of variation	df	Sum of squares	Fixation index	Percentage of variation	*P* value
Subspecies: Eastern vs. Ozark					
Among groups	1	169.9	$\Phi_{ST} = 0.54$	38.85	<0.0235
Among populations within groups	6	87.2	$\Phi_{SC} = 0.25$	15.21	<0.0001
Within populations	544	353.2	$\Phi_{CT} = 0.39$	45.94	<0.0001
Three clusters: Eastern (all populations); Ozark NF&BC; Ozark CU&EP					
Among groups	2	237.1	$\Phi_{ST} = 0.53$	48.53	<0.0029
Among populations within groups	5	20.0	$\Phi_{SC} = 0.08$	4.00	<0.0001
Within populations	544	353.2	$\Phi_{CT} = 0.49$	47.47	<0.0001

Groups were defined either by subspecies or by the three genetic clusters identified by STRUCTURE. Significance levels were determined using permutation tests (1023 permutations) in ARLEQUIN 3.1.1

range: 0.19–0.51) in a study using data from nine microsatellite loci (Mullen et al. 2010). Although our study utilized fewer loci, we found similar variation between and among populations and clear population structure. The seven loci we used, of which six are highly polymorphic, provided a mean assignment probability of >97%.

Studies of hellbenders using mitochondrial markers (Routman 1993; Routman et al. 1994; Sabatino and Routman 2009) have detected phylogeographic patterns that can aid protection and restoration efforts. Our study has demonstrated that the patterns became clearer when nuclear variation was added to the conservation arsenal. The utility of microsatellite markers was reinforced recently when Unger et al. (2010), using a different suite of microsatellite loci to examine 31 samples from a single Indiana population of *C. a. alleganiensis*, found a level of variability even higher than that observed in this study (average of 9.3 alleles/locus and observed heterozygosity values from 0.65 to 0.94). The disparity between their findings and ours may be related to the distribution of sampling areas; the Indiana population is more centrally located within the eastern hellbender range while Missouri populations are on the periphery; this may lead to the former having more exposure to hellbender diversity and gene flow. Nonetheless, the relationship between historical and contemporary drainage systems can be seen in both mitochondrial and microsatellite data and thus the combination of the two leads to the most accurate portrayal of genetic diversity.

Population structure

Multiple lines of evidence now confirm that gene flow is limited within *C. alleganiensis* in the Ozark highlands, resulting in highly isolated populations. Sabatino and Routman (2009) concluded that female-mediated gene flow was limited because they found strong mtDNA differentiation among populations, but little within-population variation. Our microsatellite results revealed a similar pattern, suggesting that neither sex has long-distance dispersal. Mark-recapture studies have confirmed that once established, adult individuals of both sexes move little within rivers, even after several months have elapsed (Nickerson and Mays 1973b). To investigate the possibility of reintroducing hellbenders to areas from which they were extirpated, Gates et al. (1985) released hellbenders and monitored dispersal, finding an average dispersal distance of 1026 m. However, dispersal depends upon the presence of large, flat rocks and swiftly flowing water (Hillis and Bellis 1971). Declining or extirpated populations are highly unlikely to be recolonized from nearby sources if the intervening habitat is inhospitable; human activities such as the installation of dams may have already severed historical dispersal routes.

Private alleles found today in six of the eight drainages we surveyed may have been shared among basins in the past but are now restricted to single populations due to reductions in population size and connectivity, and genetic drift. For the north-flowing streams of *C. a. alleganiensis*, mitochondrial sequence divergence among drainages is low (<0.3%; Sabatino and Routman 2009). Our data indicate that there is fine scale population structure among eastern hellbender populations in the northern Ozark drainages, as demonstrated by the significant genetic distance between the Big Piney River and the Meramec and Niangua Rivers, but not between the Big Piney and Gasconade Rivers. The Big Piney River is a tributary of the Gasconade River and thus gene flow is likely to have occurred in the recent geological past. For the south-flowing streams of *C. a. bishopi*, we also found strong population differentiation among all populations except the North Fork of the White River and Bryant Creek.

While genetic drift and inbreeding are important concerns for small populations, our results suggest that none of the surveyed populations currently suffer from severe genetic erosion. Heterozygosity levels are moderate across all populations, and we found relatively high numbers of alleles at six of the seven loci. It is important to conserve as much of the localized diversity as possible to better enable the species to adapt to changing environmental conditions. Our results suggest that populations in the majority of these drainages are highly divergent from one another; thus, some populations may contain locally adapted genes. Phillimore et al. (2010) revealed that local adaptations to temperature variation were associated with different spawning dates in populations of frogs (*Rana temporaria*) across the United Kingdom. While restoration and release programs may be necessary in some areas, genetic differentiation across the landscape must be considered when identifying source populations.

There also is a need for informed management decisions when conducting activities that are likely to impact hellbender habitat or jeopardize what little gene flow is naturally occurring. Changes to stream substrates through gravel runoff, and the implementation of culverts and dams may sever dispersal routes and further isolate hellbenders into extremely localized populations, leading to inbreeding. Perhaps the most important outcome of this study is confirmation of the divergence between the eastern (i.e., Black River system) and western (North Fork of the White River system) populations of *C. a. bishopi*. This split is of the same magnitude as the divergence between the two subspecies, an observation supported by Sabatino and Routman (2009). They concluded that paraphyly within *C. a. bishopi* is due to divergence between tributaries of the Black River system, as evidenced by high similarity of the North Fork of the White River and an adjacent Black River

tributary, the Spring River. Perhaps the close proximity of the two streams had influenced the biogeography of the hellbender in that area, although the hellbender is found today only in the Arkansas portion of the Spring River and thus is not latitudinally proximal to the current North Fork distribution in Missouri. This is an important distinction to be considered if supplementation of either population is to involve sharing the pool of genetic diversity. Unfortunately, we were unable to obtain samples from the Spring River as that population is likely extirpated in Missouri.

Identification to source population

A potential benefit from documenting variation at highly polymorphic loci such as microsatellites is the ability to identify of the source of individuals. The high assignment probability shown in this study suggests that these markers could be used to identify the origin of captive hellbenders at zoos and aquariums in order to evaluate their potential for future breeding programs. While Ozark samples were correctly assigned >91% of the time, assignment probabilities were considerably lower for eastern samples, and thus it may be necessary to include additional markers to increase the probability of correct assignment for this subspecies.

Taxonomic classification

Sabatino and Routman (2009) sequenced portions of three mitochondrial genes (cytochrome-oxidase I, cytochrome-*b*, NADH dehydrogenase subunit 4) for hellbenders across much of their extant range. In their phylogenetic analyses, individuals from the Eleven Point and Current River systems clustered in one clade, and the North Fork of the White River formed another clade; they did not sample Bryant Creek. *C. a alleganiensis* samples from the Big Piney/Meramec/Gasconade/Niangua samples formed a single clade. Thus, both their study and the present study based on nuclear DNA suggest that the Ozark hellbender subspecies is paraphyletic. These results will have implications for the candidacy of the Ozark species as a federally endangered species, since we have identified two evolutionarily significant units that both suffer small population sizes. A phylogenetic approach may strengthen the case for recognition of the Ozark hellbender at the species level. However, as pointed out by Sabatino and Routman (2009), the issue of how to root a phylogeny when the closest outgroup may be excessively divergent relative to the ingroup is not trivial. *C. alleganiensis* is the sole member of its genus, and Cryptobranchidae has only two genera, the second being the two giant *Andrias* spp. of China and Japan. Extensive sampling of Ozark hellbenders in Missouri and Arkansas, coupled with additional outgroup eastern hellbender samples from across the range, will facilitate a phylogenetic approach to resolving species and subspecies delineations.

Implications for conservation management

Our results suggest that hellbender populations in Missouri are comprised of three distinct genetic clusters and that current subspecies delineations do not address the existing population structure that exists within the Ozark populations. These three clusters appear to have been isolated for many generations, and may be diverging on different evolutionary paths. As such, we recommend that these hellbender populations be recognized as three separate management units: the eastern hellbender cluster (containing the Niangua, Big Piney, Gasconade, and Meramec Rivers), the White River cluster of the Ozarks (containing the North Fork of the White River/Bryant Creek watershed), and the Black River cluster of the Ozarks (comprised of the Eleven Point River/Current River watersheds).

In the absence of migration needed to recolonize and/or supplement population sizes naturally, restoration via human-mediated releases may be necessary in some situations (Trenham and Marsh 2002). Based on the limited gene flow and the lack of knowledge about past inter-basin movements, we strongly recommend that conservation efforts not include translocations of individuals between genetically differentiated drainages. In addition, the amphibian chytrid fungus (*B. dendrobatidis*) has been identified in both captive and wild populations (Briggler et al. 2007a, 2008). Translocation may introduce pathogens and diseases into unaffected locations, and could put wild populations at risk of further decline (Cunningham 1996). Release planning should incorporate data on genetic structure as well as the presence of pathogens such as the chytrid fungus to increase the odds of success in terms of both the perspectives of a state regulatory/management agency and in the evolutionary sense.

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- Populations of hellbenders are incredibly isolated
- Isolation can be the result of inhospitable habitat preventing gene flow and recolonization
- Genetic differences may result in a restructuring of taxonomy raising subspecies to the species level for hellbenders
- Conservation efforts should use phylogenetically as a tool especially in the reintroduction of captive bred specimens